Departement für Pferde
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Arbeit unter wissenschaftlicher Betreuung von
PD Dr. med. Benedikt Weber, PhD

Hemodynamic Assessment of a Murine Heterotopic Biventricularly Loaded Cardiac Transplant in vivo Model

Inaugural-Dissertation

zur Erlangung der Doktorwürde der

Vetsuisse-Fakultät Universität Zürich

vorgelegt von

Agnieszka Anna Książek

Tierärztin

von Łódź, Polen

genehmigt auf Antrag von

Prof. Dr. med. vet. Brigitte von Rechenberg, Dipl. ECVS, Referentin

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“Give it sufficient time and anything we imagine can happen.“

Zusammenfassung

Heterotopische Herztransplantationen (HHT) im Mausmodell ermöglichen es die Immunologie der Organtransplantation zu erforschen und die Entwicklung von Pharmazeutika voranzutreiben. In dieser Studie wurde ein biventrikuläres HHT Mausmodell ohne Herz-Lungen Transplantation verwendet. Die HHT's (n=13) wurden an C57BL/6J Mäusen durchgeführt mittels Anastomose der kranialen Vena Cava des Spenders und der abdominalen kaudalen Vena Cava des Empfängers, der aufsteigenden Aorta des Spenders und der abdominalen Aorta des Empfängers, sowie zwischen dem pulmonalen Stamm und dem linkem Vorhof. Es wurde eine echo- und elektrokardiografische Evaluierung durchgeführt. Die Erfolgsrate lag bei 61% und die mediane Zeit der OP betrug 190min (IQR 180-250). Die Herzfrequenz in der HHT Gruppe lag bei 355±6bpm und 418±61bpm in der Kontrollgruppe. Ein annähernd natives Öffnen- und Schliessen der Aorten- und Mitralklappe wurde beobachtet. Der mittlere Durchfluss in der Anastomose zwischen dem pulmonalen Stamm und dem linken Vorhof betrug 382±12 mm/s. Das 2D „Speckle Tracking" zeigte einen Unterschied in der Ausrichtung der Flussvektoren, die auf eine asynchrone Bewegung des linken Ventrikels schliessen lässt. Diese Resultate zeigen die mikrochirurgische Durchführbarkeit eines HHT Mausmodells unter voller Belastung, mit einer vergleichbaren hemodynamischen Leistung zum orthotropen Herz. Dieses Modell ermöglicht die Beantwortung von zellulären und molekularen Fragen im murinen Herzkreislauf.

Stichwörter: Doppelkammer Belastung Herz, Kardiovaskuläre Implantation, heterotropische Herztransplantation Maus, Mausmodell, Hemodynamische Untersuchung

Summary

Heterotopic heart transplantation (HHT) in murine models enables studies on organ transplantation immunology and pharmaceutical development. Here we assess a biventricularly loaded murine HHT model without a combined heart-lung transplantation approach. HHTs (n = 13) were performed on C57BL/6J-(H-2b) mice by anastomoses between the donors' cranial vena cava and the recipients' abdominal caudal vena cava, the donors' ascending aorta and the recipients' abdominal aorta, and the grafts' pulmonary trunk with the left atrium. An echocardiographic and electrocardiographic assessment was performed. The procedure success rate was 61%. The median duration of the surgery was 190 (IQR 180-250) min. The mean heart rate in the HHT group was 355 ± 6 bpm and 418 ± 61 bpm in the control group. A native-like closing and opening pattern of the aortic and mitral valve was observed. PW Doppler showed a flow across the anastomosis of pulmonary trunk with the left atrium, with a mean maximum velocity of 382 ± 12 mm/s. 2D speckle tracking analysis revealed differences in vector alignment, indicating an asynchronous movement of the LV, when compared to the orthotopic heart. These results demonstrate the technical (micro)surgical feasibility of a fully loaded HHT murine model with hemodynamic performance approximating the native orthotopic situation. This model may open up new options for the investigation of cellular and molecular questions in the murine cardiovascular *in vivo* system.

Keywords: Biventricular loading, Cardiovascular implant, Heterotopic heart transplantation, Murine *in vivo* model, Hemodynamic assessment

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Abstract

Background: Heterotopic heart transplantation (HHT) in rodent animal models represents an important technique enabling studies on organ transplantation immunology and pharmaceutical development. Recent investigations used nonworking HHT designs with the left ventricle (LV) bypassed in the anastomosis system. In spite of their principal success, the lack of orthogonal ventricular filling leads to myocardial atrophy. However, when focusing on the cellular and molecular mechanisms involved in the *in vivo* remodeling of the myocardium or cell-based cardiovascular implants, a nonworking model is suboptimal as it lacks the native-analogous hemodynamic and metabolic situation. Here we present the hemodynamic end electrical assessment of a biventricularly loaded murine HHT method without the need for a combined heart-lung transplantation approach.

Methods: Heterotopic transplantations (n = 13) were performed on C57BL/6J-(H-2b) inbred mice (n = 13 donors, n = 13 recipients) by creating end-to-side anastomoses between the donors' cranial vena cava (CrVC) and the recipients' abdominal caudal vena cava (CVC), between the donors' ascending aorta and the recipients' abdominal aorta (aAo), and between the grafts' pulmonary trunk and the left atrium. After transplantation, a hemodynamic assessment using echocardiography (including 2D speckle tracking analysis) and electrocardiography was performed.

Results: The loaded HHT procedure in the mice was performed with an overall success rate of 61%. In 3 of the remaining 5 cases, only atrial function was restored. The median duration of the entire surgical procedure for the recipient animal was 190 (IQR 180-250) min. The mean heart rate in the loaded HHT group was 355 ± 6 bpm in comparison to the control group with an in situ heart rate of 418 ± 61 bpm. A native-like closing and opening pattern of the aortic and mitral valve (visible on both 2D and M-mode images) was observed, confirming a native-analogous loading of the LV. Pulsed-wave Doppler provided visualization of the flow across the region of anastomoses between the pulmonary trunk and the left atrium, reaching a mean maximum velocity of 382 ± 12 mm/s. Exemplary 2D speckle tracking analysis of the LV free wall and interventricular septum revealed some differences in vector directions in one animal when compared to the orthotopic native heart, indicating an asynchronous movement of the LV.

Conclusions: These results demonstrate the technical (micro)surgical feasibility of a fully loaded HHT procedure in the murine model without using a combined heart-lung transplantation approach. The acute hemodynamic performance of the HHT grafts approximated the native orthotopic situation. This model may open up new options for the investigation of cellular and molecular questions in the murine cardiovascular *in vivo* system in the near future.

Introduction

Heterotopic heart transplantation (HHT) was developed for experimental purposes serving as an alternative technique to orthotopic transplantation, mainly because it does not require a cardiopulmonary bypass, which has not been realized in murine models so far. In addition, in case of nonorthotopic cardiac transplants the survival of the recipient does not primarily depend on the graft's postsurgical performance, which represents another major experimental advantage. Initial attempts aimed at the creation of nonvascularized heart transplants, where only cardiac tissue was implanted subcutaneously in the pinna of the mouse ear (ear-heart transplant) [1, 2]. This model, however, was unsuitable for studies on cardiac allograft vasculopathy, as it lacked coronary perfusion and therefore also the blood-endothelial interface as a main location for immunologic reactions [3]. Hence, fully vascularized HHT models were developed being primarily based on a retrograde coronary and ventricular perfusion from the abdominal aorta (aAo). This heterotopic 'unloaded' heart transplantation approach has been used in mice for experimental purposes for almost 45 years [4], and in rats for almost 50 years [5].

Since then, advances in microsurgical techniques have allowed several modifications and technical improvements of the initially described models [3, 6, 7]. Although heart transplantation in mice has been reported to be much more surgically challenging when compared with implantation in the rat model, mainly due to the difference in size, HHT in mice carries several inherent advantages. This includes the availability of a plethora of valuable experimental, transgenic, immunocompromised, and humanized models not available in other animal species such as rats. In addition, the mouse model offers a broad supply of commercially

available reagents for basic scientific evaluation as the murine system represents an immunologically and genetically well-established model.

In spite of the major scientific achievement of (retrograde) vascularization of the transplanted hearts, the model of unloaded HHTs is mainly limited to studies on transplantation immunology. This results from the limitation of hemodynamic loading to the right heart only with functional exclusion of the left heart. As a result, the myocardial mass decreases over time due to progressive myocardial atrophy [8-10].

To increase the representative value of the results obtained from HHT, the flow through the graft's left ventricle (LV) seems indispensable for an assessment of native-analogous cardiac hemodynamics and metabolism. Therefore, several research groups have investigated different approaches towards increased loading of the left ventricular side in order to create a fully loaded, 'working' HHT model [8, 11-15]. In spite of these important pioneering studies on the improvement of myocardial loading in HHT models, these approaches could only generate a native-analogous, fully loaded myocardial environment via integrating a combined heart-lung transplantation approach into the murine system [16]. With the presence of pulmonic tissue in the peritoneal cavity, the practicability and long-term functionality of these combined models are expected to be highly limited [11]. Other initial attempts have focused on the combined transplantation of the pulmonary valve and the heart for generating biventricularly loaded model systems [15]. However, a systematic hemodynamic and electrical assessment of biventricularly loaded HHT model systems in order to investigate their representative value for the native situation is missing. Therefore, the present study aims at the hemodynamic and electrical assessment of a biventricularly (fully) loaded HHT approach in the murine *in vivo* model.

Materials and Methods

Animals

Female C57BL/6J (H-2b) inbred mice (n = 26) of a mean weight of 24.01 g (SD 2.9) for the recipient group (n = 13) and 22.44 g (SD 2.3) for the donor group (n = 13) were purchased from Harlan (Hsd) Laboratories (The Netherlands) and were housed in a certified rodent facility. Before surgery was commenced, the animals were given an appropriate acclimatization time, they were fed with normal autoclaved chow diet, and they had free access to water. All animals received humane care, and the study was approved by the Cantonal Ethics Committee [State Veterinary Office of the Canton of Zurich (No. 160/2013, Int. 4922), Switzerland] in compliance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health. All precautions were taken to minimize the animals' suffering.

The animals were weighed and anesthetized with 4-5% isoflurane (induction) in oxygen (0.8-1.5 liters/min) in the induction chamber. Anesthesia was maintained with 0.5-1.5% isoflurane in 0.5-1 liter/min oxygen (mask connected to Bain's circuit). The mice were monitored with a pulse oximeter during the surgery and postsurgical survival time (6 h). For analgesic purposes, buprenorphine (Temgesic®; 0.1 mg/kg s.c.) was injected 1 h before the first skin incision. The animals were injected with saline solution (5 ml/kg/h s.c.) to maintain appropriate hydration during the entire surgery and postoperative survival time.

Surgical Procedure

All surgical procedures were performed by a single operator. Figure 1 illustrates the schematic overview of the biventricularly loaded HHT procedure, and figure 2 shows a schematic overview of blood flow

direction in the biventricularly loaded HHT procedure. The surgical procedure included the following individual steps.

Harvest of the Donor Heart

The donor animals (n = 13) were anesthetized and placed in dorsal recumbency (supine), and a midline xiphopubic laparotomy was performed using a dissecting microscope (Olympus biSZX10). The abdominal viscera were lateralized with cotton swabs to expose the caudal vena cava (CVC) and aAo between the renal vessels and iliac bifurcation. Both vessels were separated from each other and ligated caudally (close to the iliac bifurcation) with single sutures (Ethicon®; Vicryl™ 6-0). Subsequently, 3-5 ml of prepared cold NaCl-heparin solution (25 IU/ml heparin in 0.9% NaCl, 4°C; Braun®) were injected into the aAo (cranially to the ligations, using a 29-gauge needle); simultaneously, while slowly injecting the solution, CVC was cut open to exsanguinate the animal and heparinize the donor heart graft with its topical cooling. As soon as the animal was euthanized, the abdominal incision was extended cranially by a U-shaped anterior thoracotomy to expose the thoracic organs. The upper half of the thoracic cavity and the thymus were removed for better visualization. The heart was released from the surrounding tissue by ligatures (8-0, PP, YAVO) of the CVC (as close to the heart base as possible), the left CrVC with the left azygos vein, and the right and the left lung including ligation of pulmonary arteries proximal to the pulmonary trunk (PT) bifurcation and the pulmonary veins. The right CrVC, aorta, and PT were dissected from the surrounding tissue and cut open without ligating them; the right CrVC was cut far cranially from the right atrium to obtain a long stump for the anastomosis with the recipient's CVC, aorta at the level of the aortic arch just before the outflow of the brachiocephalic trunk, and PT just before its

bifurcation. The suture line of the left CrVC and around the left and the right lung hilus was kept approximately 2 cm long to allow fixation of the heart ex vivo. The graft was immediately immersed in cold 5% heparin preservation solution (4°C, Belzer UW® Cold Storage solution; Bridge for Life Ltd.) and placed on ice.

Ex vivo Procedures on the Isolated Heart

The donor's explanted heart was fixated by ligature on the left CrVC and the left and the right lung hilus and kept moist in heparin preservation solution. The aorta and left auricle (LAa) were fixed in place with the cotton pad exposing the outer, smooth side of the left atrium (LA), where the incision was made matching the size of the PT. The end-to-side anastomosis of the PT and LA was performed using simple continuous suture (Ethilon™, PP, 11-0) (fig. 3). After completion of the anastomosis, the heart was released from fixation and stored for transplantation.

Recipient Preparation

The recipient animals (n = 13) were anesthetized and placed in dorsal recumbency on the heating pad, and a midline xiphopubic laparotomy was performed as on the donor animals. Two gauzes soaked with 39°C saline were placed above and below the infrarenal surgical site, wrapping the intestines and omentum for exposure of the aAo and CVC between the renal vessels and iliac bifurcation. The parietal peritoneum and fat tissue covering major abdominal vessels were stripped, and all outflowing small vessels were cauterized and cut. The adventitia was removed from the aAo, allowing separation from the CVC, and both vessels were separated from the surrounding tissue at the vascular clamp site. Two microvascular clamps were placed at the ends of the prepared

segments, closing the flow both through the aAo and the CVC (clamps proximal to the blood flow direction were placed first).

HHT Procedure

The graft was positioned on the right side of the abdominal cavity and wrapped with gauze moistened with cold saline to keep it in hypothermic state. As the next step, a longitudinal aAo aortotomy (using a 30-gauge needle) was performed, matching the size of the donor's ascending aorta, followed by venotomy of the recipient's CVC after size approximation with the donor's CrVC. The vessels' openings were flushed with 5% heparinized saline solution to prevent clot formation. End-to-side anastomoses were performed (Ethilon™, PP, 11-0) in the following order: the recipient's CVC together with the donor's CrVC and the graft's ascending aorta with the recipient's aAo, both with a simple continuous suture pattern (Ethilon™, PP, 11-0). The direction of blood flow within the heterotopically transplanted heart is demonstrated in figure 2. Surrounding connective tissue was placed on the anastomosis sites to promote hemostasis. The vascular clamps placed distally to the blood flow on the great vessels were unclamped first, followed by release of the ones proximal to the flow. The intestines were re-placed into the abdominal cavity, and the abdomen was closed using simple continuous sutures (YAVO, PP, 6-0). Just before the complete closure of the abdominal wall, the abdominal cavity was flushed with preheated (38°C) 0.9% saline solution to exclude possible air accumulation that could impair later ultrasonographic evaluation.

Echocardiographic in vivo Analysis

General anesthesia was maintained for 6 h postoperatively. During this time (immediately after skin closure) the recipient animals underwent transabdominal ultrasonographic evaluation (Vevo 2100;

FujiFilm VisualSonics Inc., Toronto, Ont., Canada; linear array probe MS 550: 22-55 MHz). All echocardiographic analyses were performed by a single operator. The animals were positioned in dorsal recumbency and, if necessary, turned to the left lateral position.

The following views were acquired for the heterotopically transplanted hearts (the main focus was visualization of the LV and LA): (I) 2D long-axis (LAX) view of (1) the LV/left ventricular outflow tract/aorta, (2) the LV/LA, and (3) the LA/LAa/PT-LA anastomosis; M-mode at the level of the aortic valve (AoV); pulsed-wave (PW) Doppler at the level of the artificially created anastomosis between the PT and LA, and (II) 2D short-axis (SAX) view at the level of (1) the mitral valve (MV), (2) the AoV, and (3) M-mode at the level of the MV. Physiological data were collected during the sonographic investigation [heart rate, respiratory rate, electrocardiography (ECG) trace recording]. In one of the successfully performed HHT, offline 2D speckle tracking (2DST) analysis was performed to visualize the left ventricular wall motion in comparison to the donor heart in the orthotopic position prior to the start of the surgery. Longitudinal strain (base-to-apex myocardial deformation) and strain rate (base-to-apex myocardial deformation rate velocity) values were obtained (see fig. 5, 6). After survival for 6 h, the donor animals were euthanized whilst still under general anesthesia, and the grafts were collected and fixated in 4% polyformaldehyde. Additionally, 4 female C57BL/6J (H-2b) inbred mice from the donor group (the same analgesia and anesthesia protocol) were evaluated echocardiographically before they underwent surgery. These animals served as a control (in situ) group for hemodynamic evaluation of the LV. Measurements were obtained within the first hour of anesthesia.

Results

Surgical Outcome

The loaded HHT procedure in the mice was performed at an overall success rate of 61% (8/13 animals); in 3 of the remaining 5 cases (3/13 animals), only atrial function was restored, in 1 animal arterial thromboembolism was observed, and 1 animal died due to respiratory arrest. The median (IQR) duration of the entire surgical procedure for the recipient animals was 190 (180--250) min. All surgical procedure durations are summarized in table 1. The most challenging part of the surgery was the anastomosis of the PT with the LA, due to the high fragility of the LA tissue structure. In the successfully working HHTs (n = 8), the grafts began to beat within the first minute after opening of the microvascular clamps, with marked initial filling of the coronary vessels and graft color change from pale to rose pink (fig. 3). Beating was visible macroscopically (online suppl. video S1; for all online suppl. material, see www.karger.com/doi/10.1159/000446515) and was externally palpable via the abdominal wall after closure of the incision. The mean heart rate in the loaded HHT group was 355 ± 6 bpm in comparison to the control group with an in situ heart rate of 418 ± 61 bpm; however, a difference in the overall anesthesia time has to be noted between the two groups (1 h for the control group and 3-4 h for the recipient animals in the HHT group), making these parameters not fully comparable.

Echocardiographic Evaluation

2D and M-Mode (LAX and SAX Views)

The ultrasonographic evaluation was challenging to perform due to the topical ‘instability’ of the graft within the abdominal cavity and a change of its position under the gentle pressure applied by the transducer. Visualization of the contractile properties of the heart graft was achieved

in the LAX view with identification of the LV, the left ventricular outflow tract, and the correct, native-like closing and opening pattern of the AoV (visible on both 2D and M-mode images), clearly confirming loading of the LV, which is not present in the nonworking HHT models (fig. 4). It was observed that in approximately every 3rd to 4th transplanted heart's cardiac cycle, the AoV remained in a closed position (captured on both 2D and M-mode images). A native-like closing and opening pattern of the MV was also observed in SAX view (fig. 4). Echocardiographic measurements of the left ventricular area and length were obtained from the loaded HHT and the control group from the 2D LAX images. The LV measurements and subsequent calculations of volume, ejection fraction, and cardiac output are presented in table 2. It should be noted that the duration of anesthesia differed between these two groups (1 h for the control animals vs. 3-4 h for the HHT group).

PW Doppler Mode

PW Doppler provided visualization of the flow across the region of anastomoses between the PT and LA reaching a mean maximum velocity of 382 ± 12 mm/s (fig. 4). It was not possible to obtain a good probe alignment for accurate PW Doppler measurements on blood inflow velocities at the level of the tricuspid valve and MV or outflow velocities via the pulmonic valve and AoV, respectively.

2DST Analysis

Preliminary 2DST analysis of the LV free wall and the interventricular septum was feasible in one successfully transplanted heart and showed some differences in vector directions when compared to the orthotopic native heart (fig. 4), with asynchronous movement of the LV. The absolute values for strain and strain rate were lower than in the orthotopic heart, suggesting some reduction in systolic function (fig. 5, 6).

Electric Activity of the Grafts

In all HHTs, just after unclamping the great vessels, initial atrioventricular dissociation was observed in the intraoperative view, which occurred to be transient and stabilized within the first 10 min. The grafts' cardiac cycle was detectable on the ECG tracing in between the beats of the orthotropic heart (fig. 4; see table 2 for heart rate evaluation). In one animal, ECG tracing disturbances were noticed in the graft during the postsurgical evaluation; however, due to the ECG electrode positioning in the recipient animal, the interpretation of the changes in accordance with the electrical axis in HHT was not conclusive.

Discussion

This study presents a hemodynamic and electrical assessment of biventricularly loaded HHT in the mouse model without the need for a combined heart-lung transplantation technique as described in previous studies. The technique is based on the working HHT primarily established in the rat animal model [12], where a similar approach was described. While there is only a limited need for heterotopic cardiac grafts for clinical therapy, in the murine system they do play a fundamental role for experimental *in vivo* studies. In particular, research focusing on transplantation immunology, cardiac remodeling, cellular repopulation in cardiac implants (e.g. remodeling in bioengineered valve prostheses), and ischemic myocardial scar healing/remodeling as well as studies on graft tolerance/rejection are dependent on a native-analogous model to study a plethora of different physiological and pathological phenomena. At this time, the need has been addressed by using heterotopic, nonloaded heart grafts transplanted to the aAo of rodents [3-6]. Such a retrogradely perfused and vascularized heart, even though allowing organ transplantation research, cannot be used to address questions related to full native-analogous myocardial functionality and metabolic activity. This lack of functional and metabolic activity is the result of lacking volumetric loading of the left heart resulting in cardiomyocyte apoptosis and atrophy in long-term experimental settings [8-10]. Changes are observed also on the protein level, as represented by a quantitative decrease in myosin chains in the LV together with a shift in myosin isoenzyme expression (V_1 myosin isoenzyme drop) and the presence of enzymatically less active myosin ATPase (V_3) [17, 18]. Following the need for a functional (heterotopic) heart transplantation model in mice, further attempts were aimed at the possible loading of a graft's LV to increase the

mechanical work performed by the muscle and prevent its atrophy. Already early attempts at isovolumetric loading supported the importance of cardiac muscle work for the preservation of muscle protein synthesis and RNA maintenance, bringing heterotopically transplanted hearts' molecular values closer to the *in situ* ones [19]. More advanced attempts aimed at combining heart transplantation with the lung(s) and their vasculature and they were performed on both mice and rats [8, 11, 16]. This allowed for left ventricular loading and, for the first time, physiological-like movement of the semilunar and atrioventricular valves. However, the fate of the lung parenchyma, being an essential part of this model, seems to be questionable and, with time, results in deterioration of the graft [11].

The present study was aiming to overcome the abovementioned limitations of existing HHT models used in murine strains. It was based on previous experiments performed in rats and on surgical technical reports [12, 14, 15], where the working HHT model showed outcomes superior to those of the nonworking one. This included achieving almost systemic orthotopic-like blood pressures in the right ventricle and the LV, no major macroscopic enlargement of the LA, appropriate diastolic relaxation, and responsiveness to β-receptor agonist drugs similar to that of the native heart. The authors of one study [12] described the hemodynamic characteristics of these HHT as 'almost completely equal to native hearts', representing a more physiological model for cardiovascular research. A further study additionally confirmed that the histological appearance of the myocardium in this model is similar to the native heart, calling it a 'regularly shaped, unidirectional and healthy muscle' [14]. The same group stated that morphologic observations showed no significant differences in the mean weights of donor and native hearts, confirming the proof of concept study in comparison with

the atrophy occurring in nonloaded models. Encouraged by these promising results in rats, the present study demonstrated the feasibility of this concept in the murine model by hemodynamic and assessment.

Here the loaded HHT hearts were spontaneously beating at rates comparable to those of *in situ* hearts. The overall time of the surgery was longer than in transplantations performed on rats; however, the greater surgical difficulty due to the even smaller dimensions has to be noted. The present surgical technique was found to be feasible but technically demanding. Future studies have to focus on significant shortening of the time between harvesting and unclamping, which can be expected to further increase the overall success rate of the procedure.

Hemodynamic assessment was a central element of the present investigation. Similar ultrasonographic views were obtained from the HHT group and from the control animals; however, appropriate probe alignment was a major issue. It was also the limiting factor when attempting to obtain velocity values at the level of the valves using PW Doppler. Further, longer follow-up studies have to focus in more detail on obtaining these transabdominal measurements for an accurate comparison with native values in order to further support the close-to-native-analogous behavior in this 'working' HHT design. Appropriate movement of cardiac valves (comparable to the valves in the orthotopic position) was observed; however, also in previous, non-fully-loaded heart grafts, appropriate valvular movement have been observed [12, 14, 16], suggesting that future (chronic) studies will have to address the differences in valvular opening and leaflet movements between loaded and unloaded cardiac grafts. Asynchronous opening of the AoV can be explained by the intermittent lack of a pressure gradient between the two sides of the valve in this model. However, it is expected that this phenomenon might normalize with time, which will have to be

investigated in future, longer follow-up studies.

As part of the hemodynamic evaluation of the LV, lower hemodynamic values were observed in comparison to those of the control group, suggesting 'underloading' of the graft. However, a few aspects have to be taken into consideration when interpreting this data. The smaller animal of a pair was always selected as the donor animal, suggesting a smaller mass of the transplanted heart, a different duration of general anesthesia at the moment of ultrasonographic examination of the HHT group compared to the control animals, as well as the expectation that the velocity values within the grafts would be slightly lower than those in the orthotopically positioned native hearts (due to the drop in blood pressure between a recipient's ascending aorta and its abdominal part -- decreasing also the preload exerted onto the heterotopic graft). In the study performed in rats [12], additional 'loading' by injection of 2 ml of NaCl and/or clamping of the aAo or CVC was tested, showing a strong positive response of HHT hearts, resulting in an increase in systemic pressure values compared to those of in situ hearts. This technique may also improve preloading in the case of the present mouse model, which will be the aim of further investigation.

In addition to a conventional echocardiographic assessment, 2DST analyses were for the first time integrated in the study. The findings from the 2DST analyses suggest the need for further investigation into the presented model. Especially in terms of graft preservation and the timing/duration of surgery which results in optimal graft recovery, further investigations are warranted. The changes in vector alignment may be due to either limited LV free wall recovery with remaining ischemia in some of its regions or due to nonphysiological positioning of the graft within the abdominal cavity, where abdominal organs may interfere with the interposed graft, thereby impairing the LV wall

movements. Nevertheless, due to the limited data available for 2DST as part of this pilot trial, further studies focusing exclusively on this aspect are recommended. To perform reliable 2DST measurements, graft alignment must allow for an excellent-quality LV LAX view with clear endo- and epicardial borders for the appropriate measurement by the 2DST software. This, however, was difficult to achieve transabdominally in all the successfully performed transplantations - mainly due to graft instability under the pressure applied by the ultrasonographic transducer. It has to be taken into account that all graft maneuvers in the acute phase of the experiment carry a high risk of bleeding and additional graft trauma and thus have to be limited to the absolutely necessary minimum. In spite of its preliminary nature, the present 2DST analysis represents the first described (both in the unloaded and the loaded design) for an HHT procedure in mice. Further refinement of this technique will have to be addressed in longer follow-up studies when all direct postoperative risks will be excluded.

The limited surgical success rate (of 61%) is certainly a disadvantage of the study. Nevertheless, for a proof-of-surgical-concept study of such high microsurgical complexity, it still correlates well with the success rates of novel HHT techniques published. Previous studies of comparable content have reported that up to 250 surgeries should be performed before such a technique can be adequately accomplished. Some authors note that it takes an average of 11 attempts to achieve the first successful procedure [20, 21]. In light of these statements, after nearly 50 years of research on nonloaded HHT in rodents, this model of biventricular loading might induce novel experimental designs and investigations.

Another major limitation of the present study is the long overall duration of the surgical procedure with a median duration of 190 min. In particular when considering recent cardiac transplant studies on the murine model

with procedure times of about 60 min [22], the overall duration of the procedure needs to be optimized as part of future trials. This seems most important when considering that the long ischemia-reperfusion times may have significantly affected the overall success rate. By limiting the ischemia-reperfusion time, the overall procedural success rate might also be improved in future studies.

A limitation of the presented approach concerns the lack of information on the degree of oxygenation of the blood in the ascending aorta of the graft, since it is composed of arterial blood from the aAo (retrograde flow) and venous blood from the CVC (antegrade flow). However, a low degree of oxygenation would represent a significant difference when compared to the native situation and might affect the (long-term) functionality of the LV, because low-oxygenated blood would be entering the coronaries. Future studies have to address the degree of oxygenation of the blood in the ascending aorta as a potential difference from the native situation.

Following ethical guidelines on novel method establishment, results obtained from acute experiments should further stimulate research on chronic follow-up models. Thus, it should be stressed that further analyses of the proposed model should be performed in terms of diagnostic assessment methods, including not only ultrasonography but also MRI. Yet, at this point of acute analysis, it was more important to use noninvasive methods only, with no additional stress from MRI contrast enhancement and prolongation of the postoperative anesthesia time, yet still reliable data acquisition [23-25].

The present results reflect the surgical feasibility and hemodynamic assessment of HHT in mice with biventricular loading and confirm that there is no need for a combined heart-lung transplantation design. Even though the chronic hemodynamic outcome of the model is still unclear and has to be defined in further, longer follow-up studies, this murine

model may represent a significant step towards the establishment of a heterotopic heart research platform that is hemodynamically comparable to the native orthotopic situation. It may further open up a broad range of new possibilities of experimental studies, allowing scientists to address many questions in cardiovascular research, with potential for the model to be extrapolated to immunoincompetent or humanized mouse strains.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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Index of Abbreviations

2D	Two dimensional
2DST	2 Dimentional Speckle Tracking
aAo	Abdominal Aorta
AO	Ascending Aorta
AoV	Aortic Valve
bpm	Beats Per Minute
C	Celcius
CrVC	Cranial Vena Cava
CVC	Caudal Vena Cava
ECG	Electrocardiogram
h	Hour
HHT	Heterotopic heart transplantation
IQR	Interquartile Range
IU	International Unit
LA	Left Atrium
LAa	Left Auricle
LAX	Long Axis View
LV	Left Ventricle
M1	Mouse 1
M2	Mouse 2
MHz	Megahertz
MV	Mitral Valve
n	Number
NaCl	Natrium Chloride
PP	Polypropylene
PT	Pulmonary Trunk
PT-LA	Pulmonary Trunk – Left Atrium
RA	Right Atrium
RV	Right Ventricle
s.c.	Subcutaneous
SAX	Short Axis View
SD	Standard Deviation

Dedication Page

This disertation is dedicated to my wonderful family: my parents Malgorzata and Piotr, my sister Dorota and her son and my nephew Krzysztof. Without their constant support and love I would have never achieved so much in my life. They are my constant inspiration and their enthusiasm and deep belief that I can succeed in everything I do were crutial and uplifting during these three years, when „impossible became possible". I would also like to ephesise my heartfelt thank you to an extremely important person in my life – Ansgar, because of whom I started my doctoral thesis at first place. He has never stopped believing in my talents even in the moments of struggle and dissapointment and have always been my greatest inspiration. I will never forget him this and I hope that I will be able to repay and show my gratitude for it one day.

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Table 1

Operative time (min) in the working HHT model in mice.

Event	Time[1]	Time[2]
Donor heart harvesting	20 (10 - 30)	21 (17.5 – 28)
Ex vivo PT-LA anastomosis	60 (45 – 110)	60 (48 – 105)
Cardiac cold ischemia	270 (228 – 300)	262.5 (226 – 275)
Ischemia (recipient animal)	100 (90 – 107)	97.5 (89 – 102)

All values are presented as median (IQR). [1] Per total number of animals (n = 13). [2] Per total number of successfully transplanted animals (n = 8).

Table 2

TV echocardiographic measurements and calculations.

Paramete	Loaded HHT group	Control group
Heart rate, bpm	355 ± 6	418 ± 61
LV area;s, mm^2	8.6 ± 1.4	11.9 ± 4.1
LV area;d, mm^2	10.5 ± 1.5	20.9 ± 3.9
Volume;s, μl	12.5 ± 3.3	22.2 ± 11.4
Volume;d, μl	17.3 ± 4.6	52.3 ± 15.1
SV, μl	4.9 ± 1.4	30.1 ± 4.7
EF, %	28 ± 3	60 ± 13
FS, %	10 ± 4	15 ± 3
CO, ml/min	1.8 ± 0.5	12.5 ± 2.2

All values are presented as mean ± SD. Calculations were obtained according to the following formulas (from LAX LV trace): Volume;s = (7.0/(2.4 + LVID;s)) × $LVID;s^3$; Volume;d = (7.0/(2.4 + LVID;d)) × $LVID;d^3$; SV = Volume;d - Volume;s; EF = 100 × SV/Volume:d; FS = 100 × (average LVID;d - average LVID;s/average LVID;d]; CO = SV × heart rate/1,000, where LVID is a LAX line length that extends from the heart base to the farthest extend of the spline. s = Systole; d = diastole; SV = stroke volume; EF = ejection fraction; FS = fractional shortening; CO = cardiac output.

Figure 1.

Schematic overview of the biventricularly loaded HHT procedure. PA-LA = PT-LA; M1 = mouse 1; M2 = mouse 2; a = ascending aorta; b = CrVC; C = PT; RA = right atrium; RV = right ventricle; LA = left atrium; LV = left ventricle.

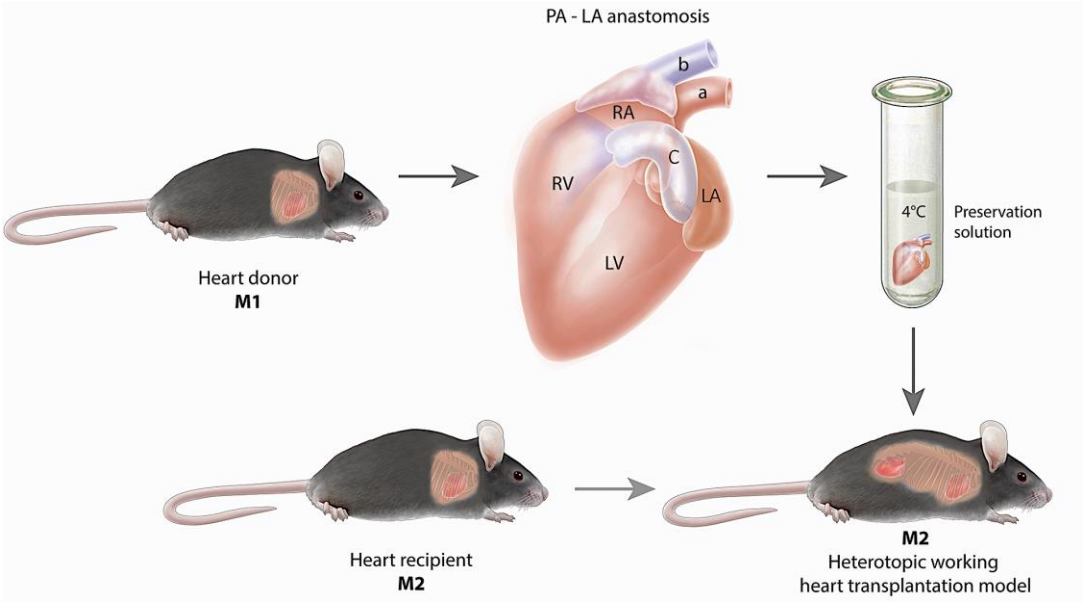

Figure 2.

Blood flow direction in biventricularly loaded HHT design. M1 = Mouse 1; a = ascending aorta; b = CrVC; C = PT; RA = right atrium; RV = right ventricle; LA = left atrium; LV = left ventricle; A = recipient's aAo; B = recipient's abdominal CVC; RBC = red blood cells; arrows = direction of blood flow.

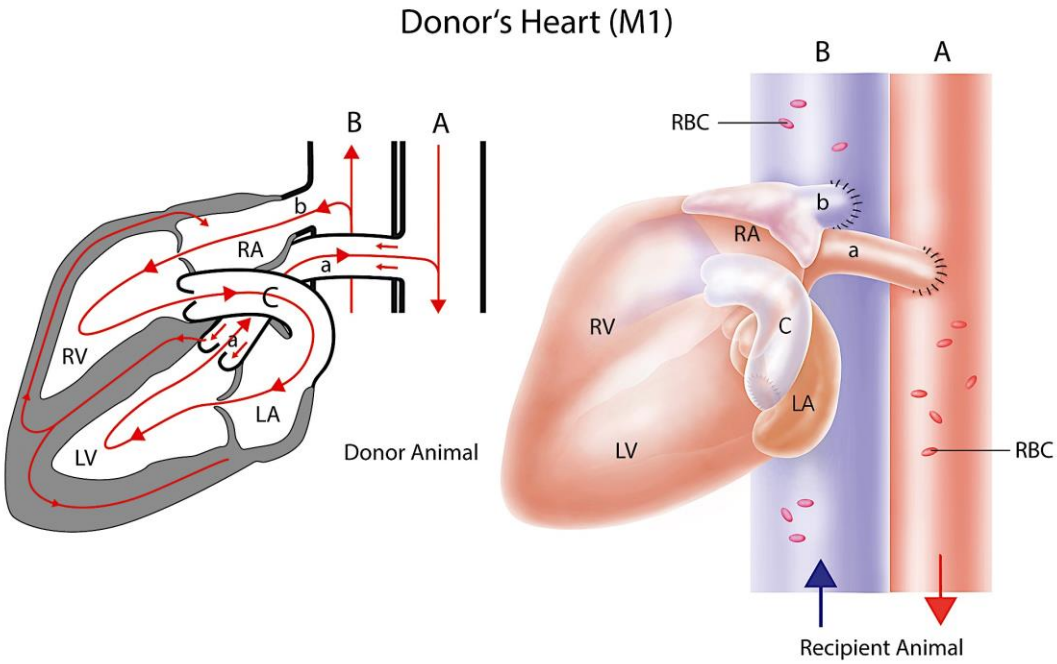

Figure 3.

Loaded HHT in a mouse, intraoperative view. Alignment of the PT after its anastomosis with the LA at the physiological position of the LAa (**a**) and after it has been unfolded for visualization of the anastomosis site (**a***). The asterisk displayed in **a*** indicates the anastomosis site. **b** Intraoperative abdominal view on anastomoses of the donor's aorta with the recipient's aAo and the donor's CrVC with the recipient's CVC. **c, c*** Heterotopically transplanted heart just at the moment of unclamping the microvascular clamps (**c**) and after the heart has been fully reperfused (**c***). Arrows: progressive filling of the coronary vessels. AO = Donor's ascending aorta, aAo = recipient's abdominal aorta, CrVC - cranial vena cava; CVC – caudal vena cava; LAa – left auricle; PT – pulmonary trunk; RA = right auricle; RV = right ventricle; LV = left ventricle.

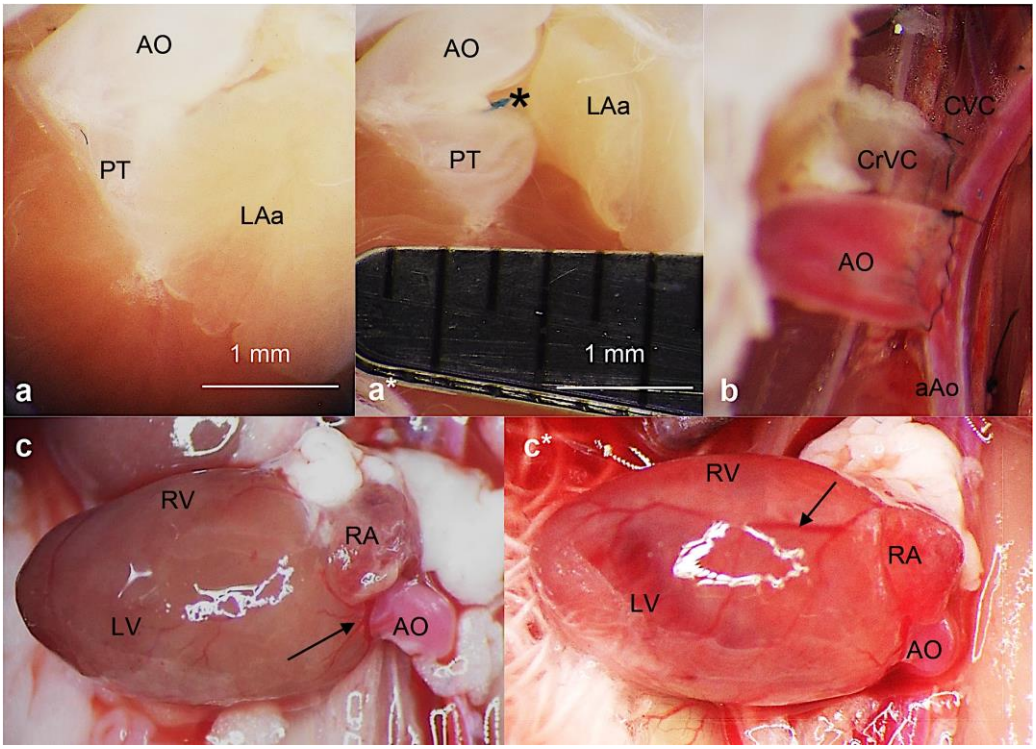

Figure 4.

Echocardiographic images of a heterotopically transplanted heart. **a** Serial B-mode images illustrating donor's heart transplanted into the abdominal cavity of the recipient mouse and changes in its LV size during one beat, with marked opening and closing of AoV (bold arrow); LAX view. ECG tracing: the recipient mouse's heart beats (QRS complexes; thin arrows) and heterotopically transplanted donor's heart beats (asterisks); some of the QRS complexes are fused. **b** Blood flow velocity through the anastomosis between the PT and LA (PW Doppler mode). **c**, **d** M-mode at the level of the AoV in SAX (**c**) and LAX (**d**) view. **e** B-mode image of the SAX view at the level of the MV showing its opening (left) and closing (right), as indicated by dotted lines. **f** 2DST echocardiography. Vectors: direction and velocity of movement of the LV free wall and interventricular septum in the orthotopic (left) and the loaded heterotopically transplanted heart (right).

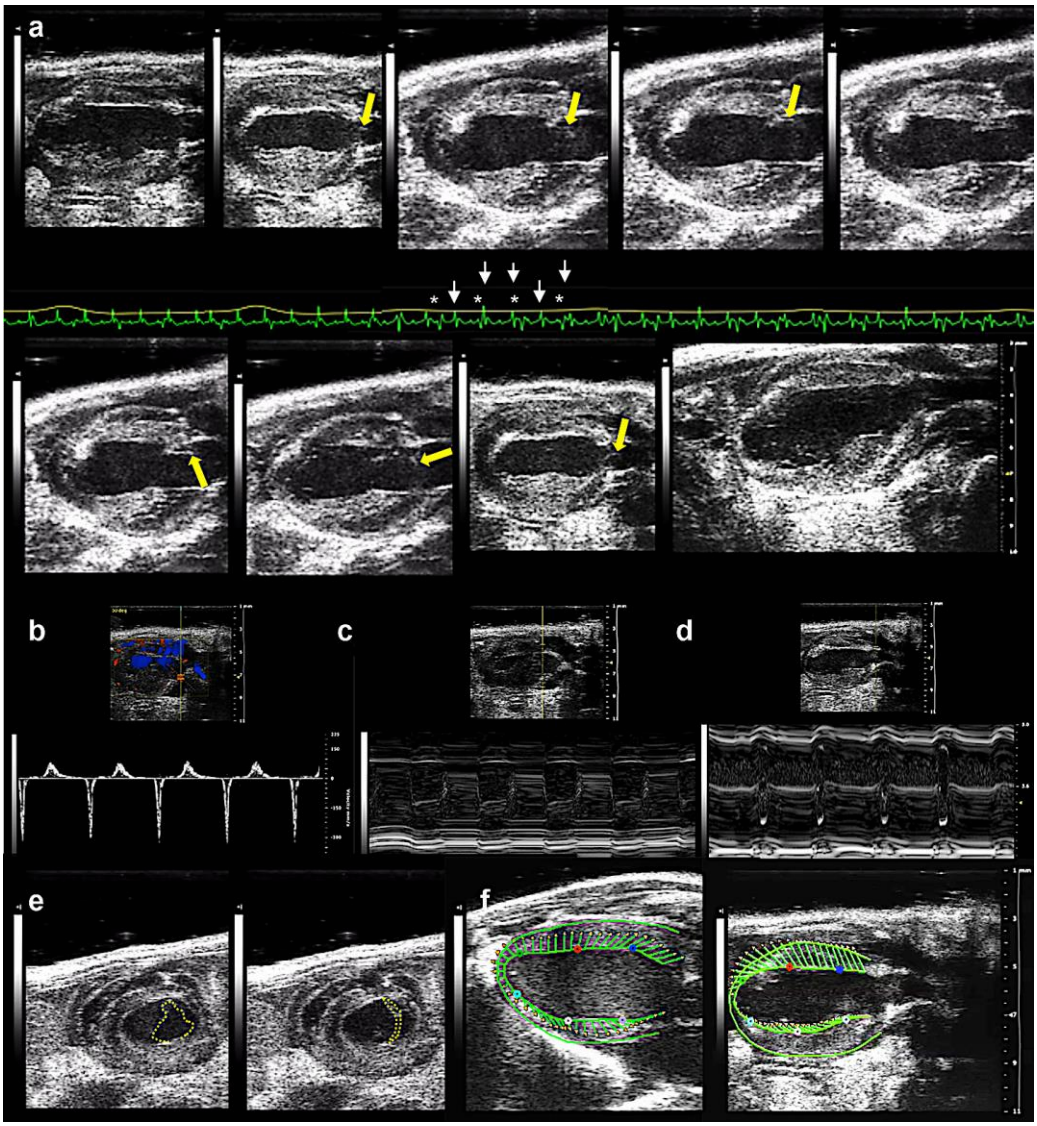

Figure 5.

2DST analysis. Comparison of longitudinal strain and strain rate graphs for the left ventricular endocardium. Comparative analysis of 2DST echocardiography of a loaded HHT (**a**, **b**) and control (**a'**, **b'**) heart obtained in a 2D LAX view. **a** Strain measure in a HHT heart. **b** Strain measure in a control heart. **b** Strain rate measure in a HHT heart. **b'** Strain rate measure in a control heart.

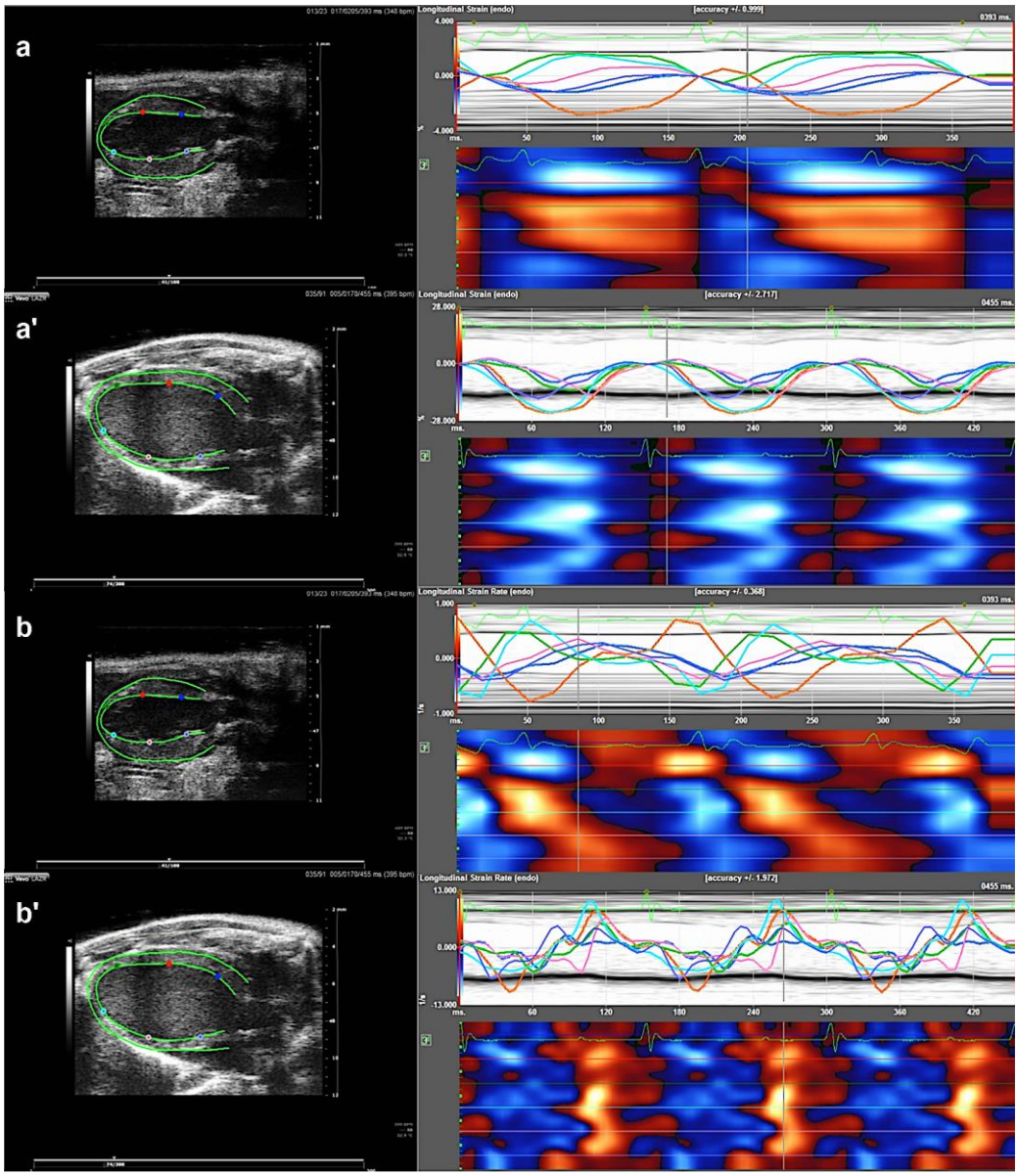

Figure 6.

Representation of the longitudinal strain rate displayed over time for the left ventrilular endocardium obtained via 2DST analysis. **a** 3D longitudinal strain rate of a loaded HHT heart. **b** 3D longitudinal strain rate of a control heart.